Identification of 19-Nor-deoxycorticosterone in Normal Rat Serum by Enzyme Immunoassay and Gas Chromatography-Mass Spectrometry

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Abstracts

Recent reports on the isolation and identification of 19-nor-deoxycorticosterone from the urine of rats with adrenal regeneration hypertension give rise to the possibility of a role of this steroid in the pathogenesis of low renin essential hypertension. The present study was undertaken to investigate the presence of 19-nor-deoxycorticosterone in normal rat serum both by a sensitive enzyme immunoassay and by gas chromatography-mass spectrometry (GC/MS).

19-Nor-deoxycorticosterone in rat serum was separated from other steroids prior to enzyme immunoassay by using high performance liquid chromatography (HPLC). The average concentration of 19-nor-deoxycorticosterone in normal rat serum was 137 ± 62 ng/dl (mean $\pm$ SD, $n=32$). Pooled normal rat serum (50 ml) was purified by HPLC and the purified sample was acetylated with acetic anhydride for GC/MS measurement. The retention time and m/z ions (358, 285, and 257) on the resulting mass fragmentogram coincided in position with those of authentic 21-acetoxy-19-nor-deoxycorticosterone and acetylated normal rat serum extract.

The combined characteristics of HPLC elution, antigen-antibody reaction, GC retention and selected ion responses provided strongly evidence supporting the presence of 19-nor-deoxycorticosterone in normal rat serum.

Previously it was reported that authentic 19-nor-deoxycorticosterone (19-nor-DOC) was from 1.5 to 5 times more potent than the parent steroid, 11-deoxycorticosterone (DOC), according to a sodium retaining bioassay (Axelrad *et al.*, 1954; Kagawa and Armen, 1954). However, the existence of 19-nor-DOC in mammals was not confirmed until

its recent discovery in the urine of adrenal regeneration hypertensive rats (Gomez-Sanchez *et al.*, 1979) and also in the urine of normal man (Dale *et al.*, 1981). Thereafter, much interest has been centered on the establishment of the physiological significance of 19-nor-DOC in adrenal regeneration hypertensive rat and in man.

Recent studies have revealed 19-nor-DOC to have a higher affinity for mineralocorticoid

receptors (Funder *et al.*, 1978). Moreover, it has been shown that this steroid caused hypertension and hypokalemia, and reduced renin activity in rat (Hall *et al.*, 1979). These studies suggest that 19-nor-DOC may possibly play an important role in causing hypertension in adrenal regeneration hypertensive rat. In this respect, a reliable method for estimating the amount of this steroid in circulating body fluid is important.

Here we report a highly sensitive enzyme immunoassay (EIA) of 19-nor-DOC and its application to the measurement of the steroid in normal rat serum. We also demonstrate the presence of 19-nor-DOC in normal rat serum by gas chromatography-mass spectrometry (GC/MS).

Materials and Methods

Calf intestinal alkaline phosphatase (E.C. 3.1.3.1) was purchased from Boehringer Mannheim GmbH, West Germany. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was obtained from Nakarai Chemical Co. Ltd., Japan. A Sep-Pak® C18 cartridge column was obtained from Waters Associates, U.S.A. A Chemcosorb 7 ODS-H column for high performance liquid chromatography (HPLC) was obtained from Chemco Scientific Co. Ltd., Japan. 19-Nor-DOC: homogeneous, colorless crystal, m.p. 134–136°C (from ether); i.r. $\lambda_{\max}$ 3500, 1700, and 1660 cm^{-1} (CHCl_3); u.v. $\lambda_{\max}$ 240 nm (log 4.2) and 19-nor-DOC 3-(0-carboxymethyl) oxime were prepared according to methods by Sandoval *et al.* (1955) and Janoski *et al.* (1974). Anti-serum to 19-nor-DOC 3-(0-carboxymethyl) oxime conjugated bovine serum albumin was raised in rabbit. The conjugation of alkaline phosphatase with 19-nor-DOC 3-(0-carboxymethyl) oxime was carried out by a method described elsewhere (Kobayashi *et al.*, 1978). Steroid free serum was prepared according to a method described by Heyns *et al.* (1967). Male Wistar rats (SPF; body weight 260–270 g) were used. The animals were housed in our laboratory for 14 days before use under a day-night lighting schedule with a 6 a.m.–6 p.m. light cycle and were allowed ad libitum access to food and water.

Blood samples were obtained from the abdominal aorta under light ether anesthesia and the sera were frozen at -20°C until assay.

Extraction and purification of 19-nor-DOC from rat serum for EIA

19-Nor-DOC in 100 μl of rat serum was extracted twice with 1 ml of dichloromethane. The organic layer was evaporated in vacuo and the residue was dissolved with 200 μl of 70 % methanol. 19-Nor-DOC in the solution was isolated from other steroids by a reversed phase HPLC equipped with Chemcosorb 7 ODS-H column (4.5 mm ID $\times$ 15 cm). The temperature of the column was maintained at 30°C with a column jacket (Model CH-1445, Systec Inc., U.S.A.). The mobile phase was methanol: water = 70:30 and the flow rate was 1 ml/min. 19-Nor-DOC fraction eluted from the column (retention time: 5.6 min) was collected in an assay tube and used as the 19-nor-DOC EIA sample following evaporation of the solvent.

19-Nor-DOC EIA procedure

Alkaline phosphatase conjugated 19-nor-DOC (100 μl) was added to the assay tube containing either extracted 19-nor-DOC sample or 19-nor-DOC standard. Anti-19-nor-DOC antiserum (500 μl) was then added to the tube which was incubated at room temperature for 1 h. Following the addition of 100 μl of anti-rabbit IgG goat antiserum, the tube was allowed to stand at 4°C for 16 h. The resulting antigen-antibody complexes were washed twice with 2 ml of distilled water. Finally, the alkaline phosphatase activity of the washed pellet was measured by the modified Kind-King method (Hansen 1966).

Sample preparation of rat serum for GC/MS measurement

A sample of 50 ml of pooled normal rat serum was extracted three times with 50 ml of dichloromethane. The dichloromethane layer was evaporated in vacuo and the residue was dissolved in 1 ml of 50 % methanol. The solution was applied to a Sep-Pak® C18 cartridge column. The column was eluted with 50 % methanol and 15 ml of the first eluate was collected. The solvent was evaporated to dryness and the residue was redissolved in 200 μl of 70 % methanol. The specimen was then applied to HPLC under the same described above. After evaporating the solvent of the 19-nor-DOC fraction, the residue

was treated with acetic anhydride at room temperature overnight. The dried residue of the acetylated product was used for GC/MS measurement.

Results and Discussion

The specificity of the antiserum used was estimated from 50 % cross reactivity of some other steroids using the present 19-nor-DOC EIA. The fifty percentage cross reactivity of DOC, 11-deoxycortisol, progesterone, corticosterone, 11-dehydrocorticosterone, and cortisol was 6.5, 4.0, 1.4, 0.17, 0.17, and 0.001 %, respectively. From the results of the specificity study, 19-nor-DOC in rat serum was separated from other steroids prior to EIA, using the HPLC shown in Fig. 1. The calibration curve for 19-nor-DOC EIA is shown in Fig. 2. The minimal amount of 19-nor-DOC detected was 4 pg/assay tube. Total recovery of 19-nor-DOC in serum was estimated from the proposed assay by adding authentic 19-nor-DOC to normal rat serum. The average recovery was 97 ± 7 % (mean $\pm$ SD, $n=6$), which was used for the correcting the serum concentration of 19-nor-DOC. Two serum samples were diluted with 19-nor-DOC free serum and their 19-nor-DOC concentrations were determined by the present method. A straight line, passing through the zero point, was obtained between the dilution and 19-nor-DOC concentration for each sample. The intra-assay and inter-assay coefficients of variation were 10.4 % ($n=5$) and 16.7 % ($n=6$), respectively. 19-Nor-DOC in normal rat serum was estimated by the proposed EIA and the average concentration of this steroid was found to be 137 ± 62 ng/dl (mean $\pm$ SD, $n=32$).

Fig. 3 shows a mass fragmentogram of authentic 21-acetoxy-19-nor-DOC and the acetylated normal rat serum extract. Monitored m/z ions were 358 (M^+), 285 (M^+-73), and 257 (M^+-101) which were characteristic

Fig. 1. Separation of authentic steroid mixtures by reversed phase HPLC: aldosterone (1), 18-hydroxycorticosterone (2), cortisol (3), 18-hydroxy-11-deoxycorticosterone (4), 11-deoxycortisol (5), corticosterone (6), 19-nor-DOC (7), DOC (8), and 17α -hydroxyprogesterone (9). The HPLC conditions were as follows: Instrument: Waters Associates Inc. Model 6000 A pump, Model 440 absorbance detector. Column: Chemcosorb 7 ODS-H, 4.5 mm ID $\times$ 15 cm. Column temperature: 30°C. Solvent: methanol/water=70/30. Flow rate: 1 ml/min. Detector: UV 254 nm, 0.02 absorbance unit of full scale.

of the structure in 21-acetoxy-19-nor-DOC. The retention time and m/z ions on resulting mass fragmentogram coincided in position with those of authentic 21-acetoxy-19-nor-DOC and acetylated normal rat serum extract. The combined characteristics of HPLC elution, antigen-antibody reaction, GC retention and selected ion responses provided strong evidence for the presence of 19-nor-DOC in normal rat serum.

Gomez-Sanchez *et al.* (1983) reported that on the 23rd day of adrenal regeneration, serum 19-nor-DOC could not be detected in either adrenal regeneration hypertensive or normal control rats which received 1 %

Fig. 2. Calibration curve for 19-nor-DOC EIA. The vertical bar indicates the standard deviation for six determinations.

Fig. 3. Mass fragmentogram of authentic 21-acetoxy-19-nor-DOC (A) and acetylated normal rat serum extract (B). GC/MS conditions were as follows: Instrument: Hitachi M-003 GC/MS spectrometer. Column: OV-101, 3 mm ID $\times$ 1 m. Column temperature: 250 $\rightarrow$ 300 $^{\circ}$ C (4 $^{\circ}$ C/min). Helium gas flow rate: 45 ml/min. Accelerating voltage: 7 kV. Ionizing voltage: 20 eV.

saline solution. From these results, they concluded that 19-nor-DOC is not a circulating steroid hormone. However, according to our chronological observation of the serum 19-nor-DOC concentration in both groups, the 19-nor-DOC concentration was significantly different before and after the operation; the steroid concentration decreased 2–8 weeks after the operation (Saeki *et al.*, 1983). This decrease in serum 19-nor-DOC may be caused by suppression of the renin-angiotensin system by sodium loading. Dale *et al.* (1981) reported that the urinary levels of 19-nor-DOC rose following ACTH stimulation in man. These data suggest that 19-nor-DOC may act as a steroidal hormone in the circulating body fluid. Although the exact mechanism for C-19 demethylation from the steroidal skeletal and metabolic pathway of this compound is not yet known, DOC and 19-hydroxy-11-deoxycorticosterone may possibly be precursors of 19-nor-DOC (Gomez-Sanchez *et al.*, 1982; Dale *et al.*, 1980).

In conclusion, we have developed EIA of 19-nor-DOC and used it for measuring the steroid concentration in normal rat

serum. We also demonstrated the presence of the steroid in normal rat serum by GC/MS.

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